

Preliminary Amendment

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Applicant(s): Lawrence P. WACKETT et al.

Serial No.: 09/898,238

Confirmation No.: 7517

Filed: 3 July 2001

For: ISOLATED AND PURIFIED DNA MOLECULE AND PROTEIN FOR THE DEGRADATION OF
TRIAZINE COMPOUNDS

Please replace the paragraph at page 1, lines 16-22, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

B2 Numerous studies on the environmental fate of atrazine have shown that atrazine is a recalcitrant compound that is transformed to CO₂ very slowly, if at all, under aerobic or anaerobic conditions. It has a water solubility of 33 mg/l at 27°C. Its half-life (i.e., time required for half of the original concentration to dissipate) can vary from about 4 weeks to 57 weeks if in soils at low concentration (i.e., less than about 2 parts per million (ppm)). High concentrations of atrazine, such as those occurring in spill sites, have been reported to dissipate even more slowly.

Please replace the paragraph at page 2, lines 1-28, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

B3 There have been numerous reports of the isolation of *s*-triazine-degrading microorganisms (see, e.g., Behki et al., J. Agric. Food Chem., 34, 746-749 (1986); Behki et al., Appl. Environ. Microbiol., 59, 1955-1959 (1993); Cook, FEMS Microbiol. Rev., 46, 93-116 (1987); Cook et al., J. Agric. Food Chem., 29, 1135-1143 (1981); Erickson et al., Critical Rev. Environ. Cont., 19, 1-13 (1989); Giardina et al., Agric. Biol. Chem., 44, 2067-2072 (1980); Jessee et al., Appl. Environ. Microbiol., 45, 97-102 (1983); Mandelbaum et al., Appl. Environ. Microbiol., 61, 1451-1457 (1995); Mandelbaum et al., Appl. Environ. Microbiol., 59, 1695-1701 (1993); Mandelbaum et al., Environ. Sci. Technol., 27, 1943-1946 (1993); Radosevich et al., Appl. Environ. Microbiol., 61, 297-302 (1995); and

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Cont
B3

Yanze-Kontchou et al., Appl. Environ. Microbiol., 60, 4297-4302 (1994)). Many of the organisms described, however, failed to mineralize atrazine (see, e.g., Cook, FEMS Microbiol. Rev., 46, 93-116 (1987); and Cook et al., J. Agric. Food Chem., 29, 1135-1143 (1981)). While earlier studies have reported atrazine degradation only by mixed microbial consortia, more recent reports have indicated that several isolated bacterial strains can degrade atrazine. For example, we previously reported the isolation of a pure bacterial culture, identified as *Pseudomonas* sp. strain ADP (Mandelbaum et al., Appl. Environ. Microbiol., 61, 1451-1457 (1995); Mandelbaum et al., Appl. Environ. Microbiol., 59, 1695-1701 (1993); and Mandelbaum et al., Environ. Sci. Technol., 27, 1943-1946 (1993)), which degraded a high concentration of atrazine (>1,000 µg/ml) under growth and non-growth conditions. See also, Radosevich et al., Appl. Environ. Microbiol., 61, 297-302 (1995) and Yanze-Kontchou et al., Appl. Environ. Microbiol., 60, 4297-4302 (1994). *Pseudomonas* sp. strain ADP (Atrazine Degrading *Pseudomonas*) uses atrazine as a sole source of nitrogen for growth. The organism completely mineralizes the *s*-triazine ring of atrazine under aerobic growth conditions. That is, this bacteria is capable of degrading the *s*-triazine ring and mineralizing organic intermediates to inorganic compounds and ions (e.g., CO₂).

Please replace the paragraph at page 6, line 21, to page 7, line 2, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

B4

The 1.9-kb *Ava*I genomic fragment includes the gene that encodes an enzyme that transforms atrazine to hydroxyatrazine, atrazine chlorohydrolase. As used herein, this gene is referred to as "*atzA*", whereas the protein that it encodes

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B4

is referred to as "AtzA". Hydroxyatrazine formation in the environment was previously thought to result solely from the chemical hydrolysis of atrazine (Armstrong et al., Environ. Sci. Technol., 2, 683-689 (1968); deBruijn et al., Gene, 27, 131-149 (1984); and Nair et al., Environ. Sci. Technol., 26, 2298-2300 (1992)). In contrast to reports that the first step in atrazine degradation by environmental bacteria is dealkylation, this suggests that biological transformation of atrazine to hydroxyatrazine may be widespread in natural systems.

Please replace the paragraph at page 25, lines 21-23, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

B5

^aDetermined by TLC analysis according to procedures described in the materials and methods. Unlabelled desisopropylatrazine and desethylatrazine had R_f values of 0.79 and 0.83, respectively.

Please replace the paragraph at page 26, lines 16-25, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

B6

Protein Characterization. Protein subunit sizes were determined by SDS polyacrylamide gel electrophoresis by comparison to known standard proteins, using a Mini-Protean II gel apparatus (Biorad, Hercules, CA). The size of the holoenzyme was determined by gel filtration chromatography on a Superose 6 HR (1.0 x 30.0 cm) column, using an FPLC System (Pharmacia, Uppsala, Sweden). The protein was eluted with 25 mM MOPS buffer (pH 6.9) containing

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0.1 M NaCl. Proteins with known molecular weights were used as chromatography standards. Isoelectric point determinations were done using a Pharmacia Phast-Gel System and Pharmacia IEF 3-9 media. A Pharmacia broad-range pI calibration kit was used for standards.

Please replace the paragraph at page 35, lines 14-28, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

B7

Several lines of evidence support the conclusion that the designated ORF constitutes the atrazine chlorohydrolase gene: 1) *E.coli* transformed with pMD4, gained the ability to degrade atrazine as demonstrated by clearing zones surrounding colonies on solid media containing crystalline atrazine, 2) the dechlorination activity was abolished by transposon *Tn5* insertions specifically within the 1.9-kb *AvaI* fragment and the *Tn5* insertion was located within the ORF, 3) there is also significant homology between the *atzA* ORF (40.987% identity over 484 amino acid residues) and a protein from *Rhodococcus corallinus* NRRL B-15444R which possesses an analogous catalytic activity, a triazine hydrolase which is responsible for the deamination of melamine (2,4,6-triamino-1,3,5-triazine) and dechlorination of deethylsimazine. While no typical *E. coli* -10 sequence was seen preceding the predicted start of *AtzA*, a potential *Pseudomonas* ribosome binding site was found 11 base pairs upstream of the ATG (V. Shingler et al., *J. Bacteriol.*, 174, 711-724 (1992)). This is interesting given the fact that *atzA* was expressed in *E. coli*.
